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Limb Development in a "Nonmodel" Vertebrate, the Direct-Developing Frog *Eleutherodactylus coqui*

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ABSTRACT Mechanisms that mediate limb development are regarded as highly conserved among vertebrates, especially tetrapods. Yet, this assumption is based on the study of relatively few species, and virtually none of those that display any of a large number of specialized life-history or reproductive modes, which might be expected to affect developmental pattern or process. Direct development is an alternative life history found in many anuran amphibians. Many adult features that form after hatching in metamorphic frogs, such as limbs, appear during embryogenesis in direct-developing species. Limb development in the direct-developing frog *Eleutherodactylus coqui* presents a mosaic of apparently conserved and novel features. The former include the basic sequence and pattern of limb chondrogenesis, which are typical of anurans generally and appear largely unaffected by the gross shift in developmental timing; expression of *Distal-less* protein (Dlx) in the distal ectoderm; expression of the gene *Sonic hedgehog* (*Shh*) in the zone of polarizing activity (ZPA); and the ability of the ZPA to induce supernumerary digits when transplanted to the anterior region of an early host limb bud. Novel features include the absence of a morphologically distinct apical ectodermal ridge, the ability of the limb to continue distal outgrowth and differentiation following removal of the distal ectoderm, and earlier cessation of the inductive ability of the ZPA. Attempts to represent tetrapod limb development as a developmental "module" must allow for this kind of evolutionary variation among species. *J. Exp. Zool. (Mol. Dev. Evol.) 291:375–388, 2001.*

The role of developmental processes in mediating phenotypic evolution has been the subject of intense study for the last 20–25 years, reprising an intellectual preoccupation with this subject that has recurred many times throughout the history of biology (Burian, 2000; Hall, 2000). Among the most unexpected results to emerge from the growing number of recent empirical studies is the general observation that extensive phenotypic diversity among organisms has been achieved despite extensive conservation of underlying genetic and developmental mechanisms (Gerhart and Kirschner, '97). The concept of modularity offers a potential solution to this apparent paradox (Wagner and Altenberg, '96; Kirschner and Gerhart, '98; Bolker, 2000). Specific developmental

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events and genetic regulatory processes are largely conserved within discrete developmental networks or sets of interactions, which are recombined or redeployed in toto in new or unusual developmental contexts, thereby yielding morphological and/or functional diversity (Raff, '96; Schlosser and Thieffry, 2000).

Modularity is a well-accepted paradigm in cell and developmental biology (e.g., Hartwell et al., '99). Many specific modules are known in considerable detail (Raff, '96), although mostly from a small number of "model" organisms. The prominent role of modularity in the evolution of development, however, is largely speculative. While modularity offers considerable promise as an explanatory and analytical tool (Ancel and Fontana, 2000; Klingenberg and Zaklan, 2000; Carroll, 2001; and aforementioned additional references), surprisingly little is known regarding the evolutionary fate or developmental variability of individual modules in particular clades. Yet, it is just these kinds of data from a wide range and number of developmental systems that are needed to comprehensively define the role and extent of modularity in the evolution of development. In this paper, we summarize our initial attempts to assess the evolutionary variability of one well-known developmental module, the vertebrate limb.

Beginning with the pioneering experiments by Saunders and colleagues in the 1940s (e.g., Saunders, '48) and continuing to the present day, the vertebrate limb has emerged as a model system for studying pattern formation during development (Summerbell, '74; Hinchliffe and Johnson, '80; Tickle, '95; Johnson and Tabin, '97). The limb also offers excellent opportunities to address the role(s) of developmental processes in organismal evolution, including modularity (Raff, '96; Shubin et al., '97; Kirschner and Gerhart, '98). Much of the current interest in limb development and evolution stems from recent discoveries regarding underlying cellular and molecular mechanisms, especially as revealed in the two best-known laboratory species, the chicken and the mouse. The gene *Sonic Hedgehog* (*Shh*), for example, is expressed within the limb bud in the zone of polarizing activity (ZPA; Riddle et al., '93; Pearse and Tabin, '98), where it has been implicated in anteroposterior limb patterning (Tickle, '96). Many other genes, including the *Distal-less* family (*Dlx*; Dolle et al., '92; Beanan and Sargent, 2000; Zerucha and Ekker, 2000) and several fibroblast growth factors (FGFs; Martin, '98), are expressed in distal limb bud ectoderm, which forms a distinct apical ectodermal ridge (AER) in many vertebrates (Ferrari et al., '95). The AER plays a critical role in mediating proximodistal limb development (Mahmood et al., '95; Niswander, '96) and has been found in the majority of vertebrates in which it has been sought (Hanken, '86), including the metamorphic frog *Xenopus laevis* (Tarin and Sturdee, '71).

Cellular and molecular mechanisms that mediate limb development are generally regarded as highly conserved among vertebrates (e.g., Shubin et al., '97; Martin, '98). Yet, there are relatively few studies of other species that are comparable to those published for the chicken and the mouse, and which would allow a more rigorous assessment of the evolutionary conservation or lability of particular features or of the limb development "module" overall. The dearth of information is especially problematic for anamniotes, i.e., fishes and amphibians. Despite recent studies of a few key species, such as zebrafish (Akimenko and Ekker, '95; Laforest et al., '98; Schauerte et al., '98), *Xenopus laevis* (Christen and Slack, '97, '98), and axolotl (Gardiner et al., '95; Torok et al., '98), these vertebrates remain relatively unexamined. This is especially true for the many species that display any of a large number of specialized life-history or reproductive modes, which might be expected to affect developmental pattern or process (Elinson, '87; Hanken, '92, '99; Raff and Wray, '89).

For the last several years, we have been examining morphological and molecular aspects of limb development in the Puerto Rican direct-developing frog, *Eleutherodactylus coqui*. Most species of frogs have two successive posthatching life-history stages, a herbivorous, aquatic larva and a carnivorous, terrestrial adult, which are separated by a discrete metamorphosis. Direct-developing species, however, bypass the free-living larval stage and develop directly into adults (Hanken, '92, '99; Elinson, 2001; Fig. 1). Many adult features that form only after hatching in metamorphic anurans, such as the limbs, instead form during embryogenesis in direct developers (Elinson, '90, '94). This gross change in the relative timing of development is accompanied by more subtle heterochronies (Callery and Elinson, 2000, 2001). Onset of limb formation in direct developers, for example, coincides much more closely with the initiation of neural and axial skeletal development than it does in metamorphosing taxa, in which limb development occurs much later (Hanken et al., '92; Schlosser and Roth, '97; Schlosser, 2001). Consequently, at least with respect to these features,

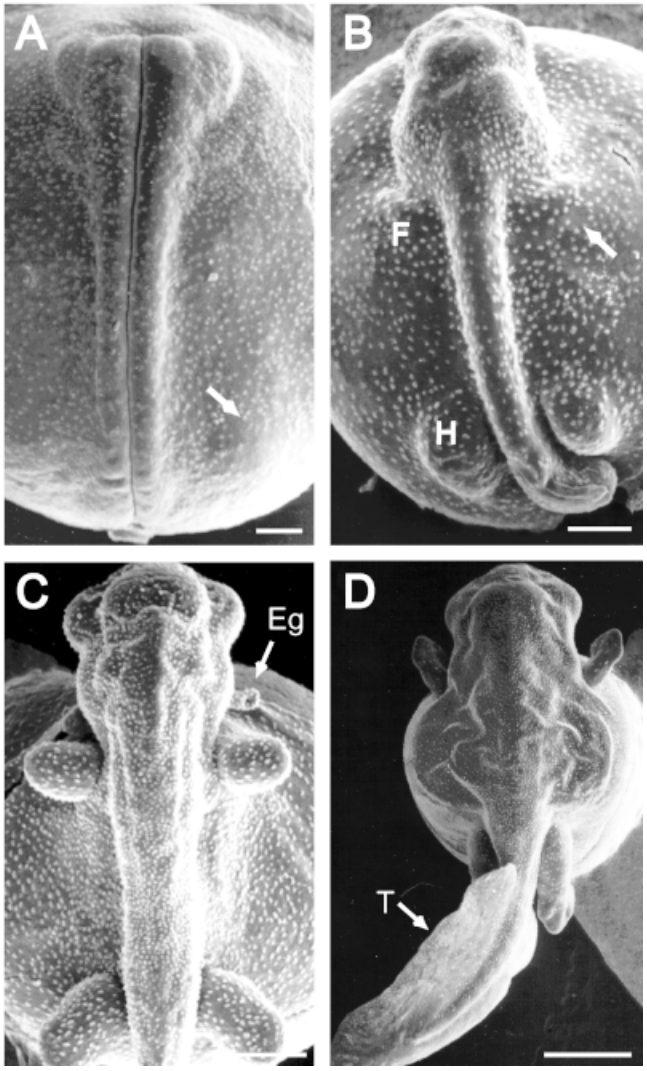

Fig. 1. Scanning electron micrographs of embryos of *E. coqui*. All specimens are shown in dorsal view, anterior is towards the top. **A**: Townsend-Stewart ('85) stage 3. Hind limb buds (arrow) are first visible as paired swellings in the dorsal ectoderm on either side of the neural folds. **B**: Stage 5. Forelimb buds are beginning to form (F, arrow). H, hind limb. **C**: Stage 6. All four limb buds are beginning to flatten dorsoventrally. Eg, external gills. **D**: Stage 13. Digits can be discerned on all four limbs. The prominent tail (T) will soon begin to regress; it will be nearly absent at hatching (stage 15). Scale bar: A, B, 0.5 mm; C, D, 1 mm.

limb development in direct-developing anurans resembles that in amniotes much more closely than it does limb development in other (metamorphosing) frogs.

Limb development in *E. coqui* is already known to differ from that in most other limbed vertebrates in one significant respect: there is no recognizable AER at any stage (Richardson et al., '98). In the present study, we extend this earlier morphological analysis by describing the sequence of limb chondrogenesis in *E. coqui* and comparing it to the sequence observed in metamorphosing anurans. To begin to characterize basic molecular features of limb development in *E. coqui,* we define the patterns of *Sonic hedgehog* (*Shh*) gene and *Distal-less* (Dlx) protein expression during embryogenesis. We focus initially on *Distal-less*, among other genes that are known to be expressed in distal limb ectoderm, because of its well-established role in mediating limb initiation and continued outgrowth in general (references follow) and because of an initial published account of *Distal-less* gene expression in *E. coqui* limbs (Fang and Elinson, '96). Finally, we use experimental embryology to begin to assess the ability of the presumptive ZPA region in *E. coqui* limb buds to mediate skeletal patterning. Results are interpreted in light of the extensive model for vertebrate limb development derived principally from the study of amniotes, and are used to assess the degree of evolutionary conservation of the developing vertebrate limb as module.

MATERIALS AND METHODS

Animals

Embryos of *E. coqui* were obtained following spontaneous mating of wild-caught adults maintained as a laboratory colony at the University of Colorado at Boulder (Elinson et al., '90). Embryos were staged according to the table of Townsend and Stewart ('85), which defines 15 embryonic stages from fertilization (1) to hatching (15). Animal collection and care were performed in accordance with the regulations of the Puerto Rico Department of Natural Resources and the University of Colorado at Boulder.

Scanning electron microscopy (SEM)

Specimen fixation, preparation, and observation with SEM followed standard procedures (Olsson and Hanken, '96).

Chondrogenesis

Embryos were fixed in Dent fixative (Dent et al., '89) and run through a graded series of ethanol baths to acid alcohol (1% hydrochloric acid in 70% ethanol). They were immersed overnight in 0.03% Alcian blue in acid alcohol, differentiated for 24 hr in acid alcohol, and dehydrated with 100% ethanol. After clearing with methyl salicylate, limbs were dissected free and examined with substage illumination.

Cloning and sequencing

The *Sonic hedgehog* (*Shh*) gene from *E. coqui*, *EcShh,* was cloned using RT-PCR. RNA was extracted from a stage 4–5 embryo (with yolk removed) and then reverse-transcribed using random hexameres and Superscript II. PCR was performed with CCCCTCTCGCCTATAAGCAGT (corresponding to bp 122–142 of *X. laevis Shh*) as the upstream primer and CGCCACTGAGTTCTCTGCTTT (corresponding to the reverse complement of bp 559–579 of *X. laevis Shh*) as the downstream primer. PCR (with 1.5 mM MgCl2) was then performed, first for 5 min at 94°C, then for 35 rounds at 94°C (45 sec), 43°C (60 sec), and 72°C (120 sec), and finally for 5 min at 72°C. The amplified fragment was cut from the gel, purified, and blunt-end cloned into the EcoRV site of BluescriptKS. Both strands of the *EcShh* were sequenced.

In situ hybridization

To assess the extent and location of *Shh* gene expression, we used a digoxigenin-labeled RNA probe to perform in situ hybridization on both whole-mount embryos (stages 3, 3+, 4–, 5–, and 6) and Paraplast (Oxford Labware, St. Louis, MO) sections (10 μ; stages 5–, 6, and 9). Hybridization generally followed the protocol of Hemmati-Brivanlou et al. (’90) and Harland (’91), and sections were prepared using standard techniques (Presnell and Schreibman, ’97).

Immunohistochemistry

Immunohistochemistry was performed using the Dlx antibody following standard procedures (Klymkowsky and Hanken, ’91; Carl and Klymkowsky, ’99; see also http://spot.colorado.edu/~klym/methods.html).

Tissue ablation and transplants

Embryos were de-jellied either chemically (2% cysteine, buffered to pH 7.8–8.0 with 5N NaOH) or manually with watchmaker’s forceps and then placed in Petri dishes with a 2% agar bed and immersed in Holtfreter antibiotic (gentamycin, 80 mg/l, in 10% Holtfreter solution) (Hamburger, ’60). Donor embryos were further immersed in Holtfreter antibiotic plus 1% aqueous neutral red (1:500 dilution) to more easily see the transplanted tissue. Immediately before surgery, embryos were anaesthetized in 0.03% aqueous TMS (ethyl m-aminobenzoate tricaine methanesulfonate; Sigma Chemical Co., St. Louis, MO, # A-5040) for as long as 15 min, and then transferred to fresh Holtfreter antibiotic.

Hind limb bud explants were removed from donor embryos by using tungsten needles or watchmaker’s forceps. Each explant was grafted to a host embryo by first making a small incision in the host hind limb bud, then removing a same-sized portion of the bud, and finally placing the explant in the incision. Grafts were immediately covered with a sliver of glass from a broken cover slip and allowed to recover in a darkened incubator at 23°C. Specimens were transferred to Holtfreter antibiotic and maintained in a darkened incubator at 23°C until being fixed overnight in MEMFA (0.1M MOPS buffered to pH 7.4 with 5 N NaOH, 2 mM EGTA, 1 mM MgSO4, and 3.7% formaldehyde) at 4°C. Fixed specimens were prepared as cartilage-stained whole-mounts with Alcian blue (Klymkowsky and Hanken, ’91).

RESULTS

Limb chondrification

Sixteen embryos were examined between stages 8 and 14 (Table 1; Fig. 2). In general, limb chondrification proceeds in a proximodistal sequence: pectoral and pelvic girdles chondrify first and distal phalanges last. Within the manus and pes, there is a posteroanterior gradient in digit formation, i.e., digits III and IV chondrify first, whereas digit I chondrifies last. Cartilage development begins slightly earlier in the hind limb than in the forelimb, but this difference is less than one embryonic stage.

Sequencing

By using RT-PCR of extracted mRNA, we isolated a 416-base-pair (bp) fragment of the *E. coqui Sonic hedgehog* gene, *EcShh* (Genbank accession number AF113403). The fragment, which corresponds to bp 143–558 of the *X. laevis Shh* gene, is 83%, 81%, 80%, and 79% similar in base-pair sequence to homologous *Sonic hedgehog* sequences in *X. laevis*, human, chicken, and zebrafish, respectively (as assessed by BLAST).

Embryonic expression of the **Sonic hedgehog *clone* EcShh**

The *Sonic hedgehog* clone *EcShh* is already expressed in axial mesoderm (notochord and prechordal plate) early in stage 3, before neural tube closure. Expression in the floor plate of the neural tube begins towards the end of stage 3, just after neural tube closure. These sites of expression are maintained into stage 5 (Fig. 3), when the gene is also expressed in the stomodeum,

TABLE 1. Limb chondrification sequences in E. coqui[1]

Limb region	Forelimb	Stage	Hind limb	Stage
Limb girdles	Pectoral girdle	8	Pelvic girdle	8
Stylopodium	Humerus	8	Femur	8
Zeugopodium	Radius	8	Tibia	8
	Ulna	8	Fibula	8
Basipodium	Radiale	9	Fibulare	8
	Carpals II–IV	9	Tibiale	8
	Ulnare-intermedium	9	Tarsals II–III	9
	Carpal I	11	Tarsal I	13
	Centrale	11	Centrale	13
	Prepollex	11	Prehallux	13
Metapodium	Metacarpals II–IV	8	Metatarsals III-V	8
	Metacarpal I	11	Metatarsal II	9
			Metatarsal I	10
Acropodium	Proximal phalanx III	8	Proximal phalanx IV	8
	Proximal phalanx IV	9	Proximal phalanges III, V	9
	Proximal phalanx II	11	Mid-proximal phalanx IV	9
	Middle phalanx III	11	Proximal phalanx II	11
	Middle phalanx IV	13	Mid-distal phalanx IV	11
	Proximal phalanx I	13	Middle phalanx V	11
	Distal phalanges II–IV	13	Middle phalanx III	13
	Distal phalanx I	14	Distal phalanges I–IV	13
			Proximal phalanx I	13

[1]Numbers indicate the Townsend-Stewart ('85) embryonic stages at which Alcian-blue staining is first detected in cleared whole-mounts. Hatching typically occurs during stage 15.

foregut, and cranial neural tissue. In the limbs, *EcShh* is first detected early in stage 5 in a crescent-shaped domain along the posterior margin of each fore- and hind limb bud. Expression in the limbs persists into stage 6, although these later domains are somewhat smaller and narrower than those at stage 5. Expression of *EcShh* disappears from the limbs and notochord by stage 9, although it is still seen in the brain, floor plate, and foregut.

Distal-less *protein expression during limb development*

Distal-less (Dlx) protein is expressed in all limb buds as soon as they are morphologically distinct: stage 3 in the hind limb, and stage 5 in the forelimb. By stage 5, Dlx expression is strongest in distal ectoderm along the margin of each limb bud (Fig. 4A–C). Dlx staining is less intense by stage 6, when the protein appears more diffusely distributed (Fig. 4D). No Dlx protein is detected in the limbs at either stage 7 or early stage 8 (Fig. 4E). Dlx is again expressed in the limbs beginning late in stage 8, coincident with the onset of morphological differentiation of the digits (Fig. 4F, I, J). The protein is present as a continuous band in the distalmost ectoderm and mesenchyme, both in the presumptive digits and between digits. Subsequently, the protein is detectable in distal parts of the digits, in both ectoderm and mesenchyme (Fig. 4G, K), but it is conspicuously absent from the ectodermally derived adhesive pads (Fig. 4H, M). Dlx protein continues to be expressed in these locations up to stage 13.

ZPA ablation and transplantation

Ablating the presumptive zone of polarizing activity (ZPA) from the hind limb bud at stages 3–6 resulted in death of the embryo (N = 34), loss of limb skeletal elements (N = 8), or loss of the entire limb (N = 6). When the region was transplanted to the anterior portion of host limb buds of the same stage between stages 3 and 5 (Fig. 5A, B), outgrowths from the implantation site were detected in all surviving embryos (N = 68), although fewer than a third of these embryos survived past stage 9 (N = 19). Specimens that survived to stage 15 (hatching) display supernumerary digits (Table 2). Digit I is the most common duplication, seen in 11 of 14 specimens (Fig. 5C). Digits I and II are both duplicated in seven specimens, and digits I, II, and III are all duplicated in two specimens. One additional specimen appears to have duplicate digits II and III but lacks normal digit I (Fig. 5E). This specimen and one other also have an extra (i.e., third) long bone that lies between the tibia and fibula in the zeugopodium (Fig. 5E), and one has a duplicate femur (not illustrated).

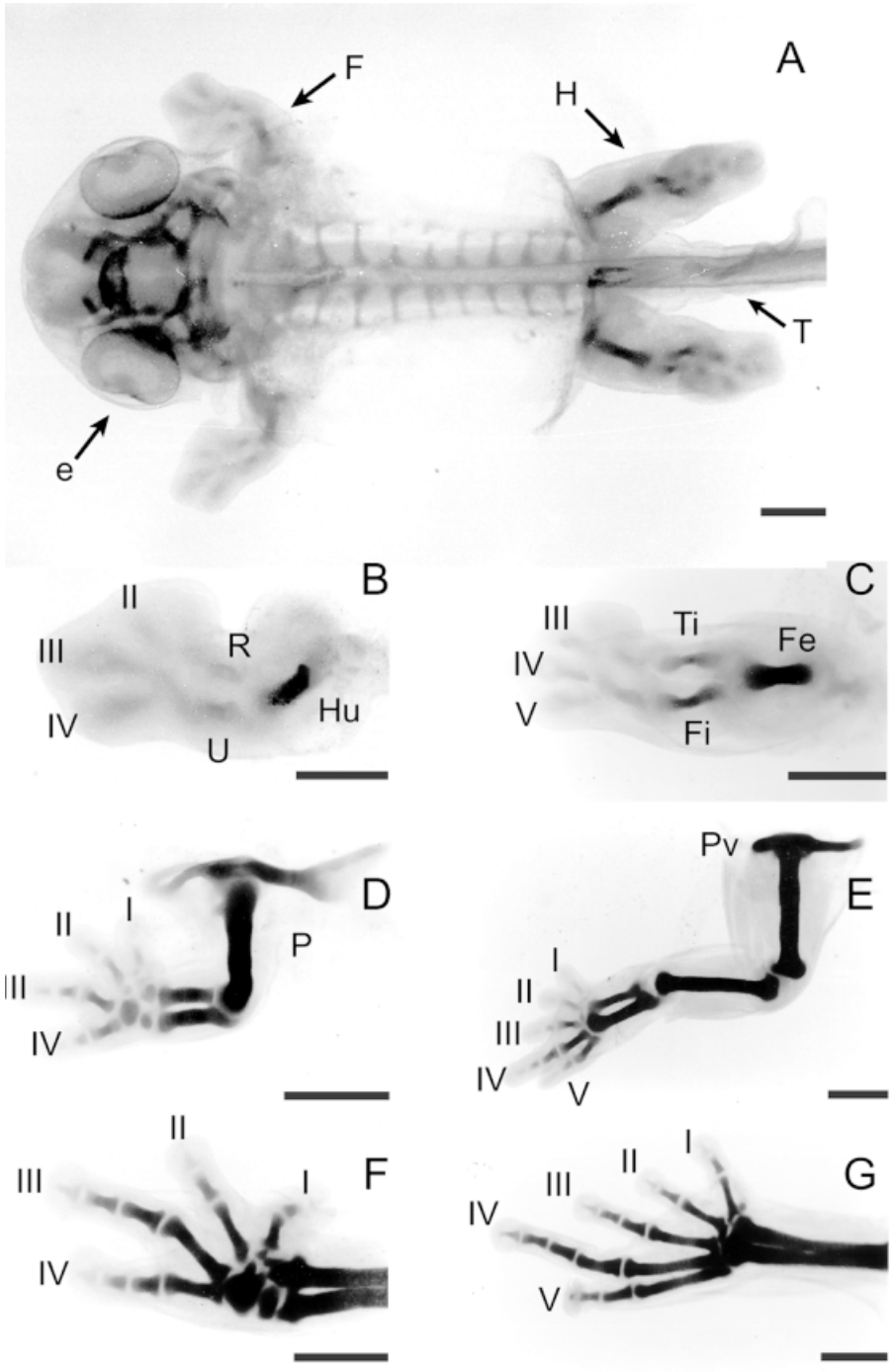

Fig. 2. Normal skeletal (cartilage) morphology in cleared and stained whole-mount embryos. All are dorsal views, B–G are left limbs; digits are labeled I–V. **A**: Stage 8, yolk sac removed. e, eye. **B**: Forelimb, stage 8. Hu, humerus; R, radius; U, ulna. **C**: Hind limb, stage 8. At this stage, the femur (Fe) is slightly more developed than the humerus (cf. panel B). Fi, fibula; Ti, tibia. **D**: Forelimb, stage 11. P, pectoral girdle. **E**: Hind limb, stage 11. Pv, pelvic girdle. **F**: Forelimb, stage 13. **G**: Hind limb, stage 14. Additional abbreviations as in Fig. 1. Scale bar, 0.5 mm.

Supernumerary digits did not form following ZPA transplants made at stage 6 (N = 5), but skeletal elements in specimens that survived to hatching are slightly distorted. In a separate control experiment, stage 6 donor ZPAs were transplanted into stage 4 host limb buds (N = 2). Supernumerary digits formed in one of these specimens. Finally, as a control for tissue specificity, similar-sized por-

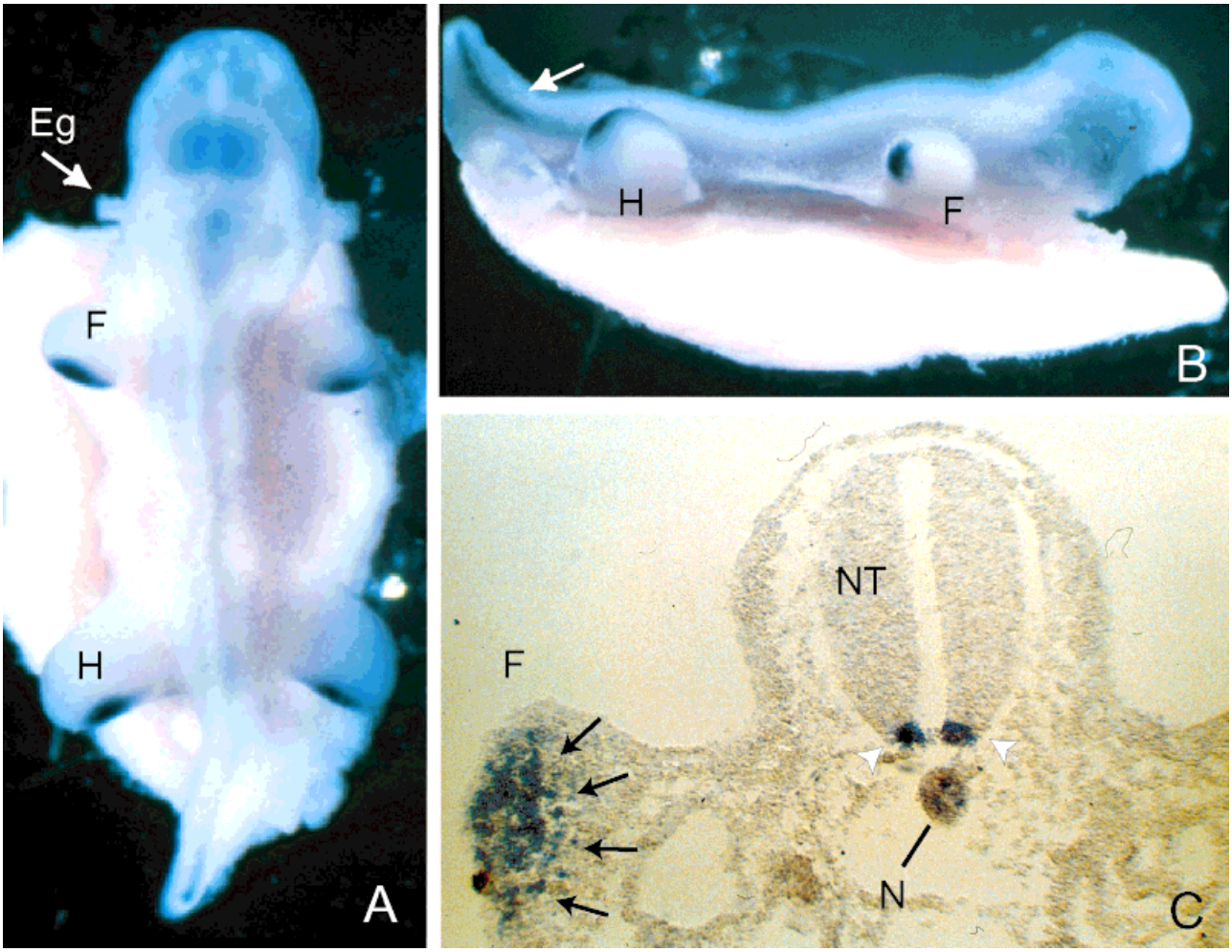

Fig. 3. Expression of the *Sonic hedgehog* clone *EcShh* in stage-5 embryos of *E. coqui*. **A**: Whole-mount in situ hybridization seen in dorsal view; anterior is at the top. *EcShh* is expressed along the posterior margin of each limb bud in a region that corresponds to the zone of polarizing activity. **B**: The same embryo seen in lateral view. *EcShh* is also expressed in the notochord, especially in the tail (arrow). **C**: Cross section of a second embryo; dorsal is at the top. *EcShh* is expressed in the floor plate of the neural tube (NT; white arrowheads), in the notochord (N), and in limb mesenchyme (black arrows). Additional abbreviations as in Fig. 1.

tions of tail ectoderm (instead of the presumptive ZPA region) were grafted into host limb buds at stage 4 (N = 3). No supernumerary digits formed in any of these grafts.

DISCUSSION

In this study, we address two principal questions. First, to what extent does embryonic limb development in direct-developing anurans conform to the model of tetrapod limb development derived principally from the study of amniotes? Second, are there any differences in limb development between metamorphosing anurans and direct-developing *Eleutherodactylus* that are correlated with the evolution of this phylogenetically derived life history and reproductive mode? Our study is among the first to assess morphological and genetic features of limb development in a direct-developing frog (see also Elinson, '94; Richardson et al., '98). Moreover, there is surprisingly little comparable data from metamorphosing species, which retain the presumed ancestral life history, especially in comparison to that available for amniotes and urodeles. Hence, the following discussion is preliminary. The difficulty in drawing robust conclusions regarding the evolution of limb development in anurans, as well as other amphibians, underscores the need for additional data from several more metamorphic and direct-developing species.

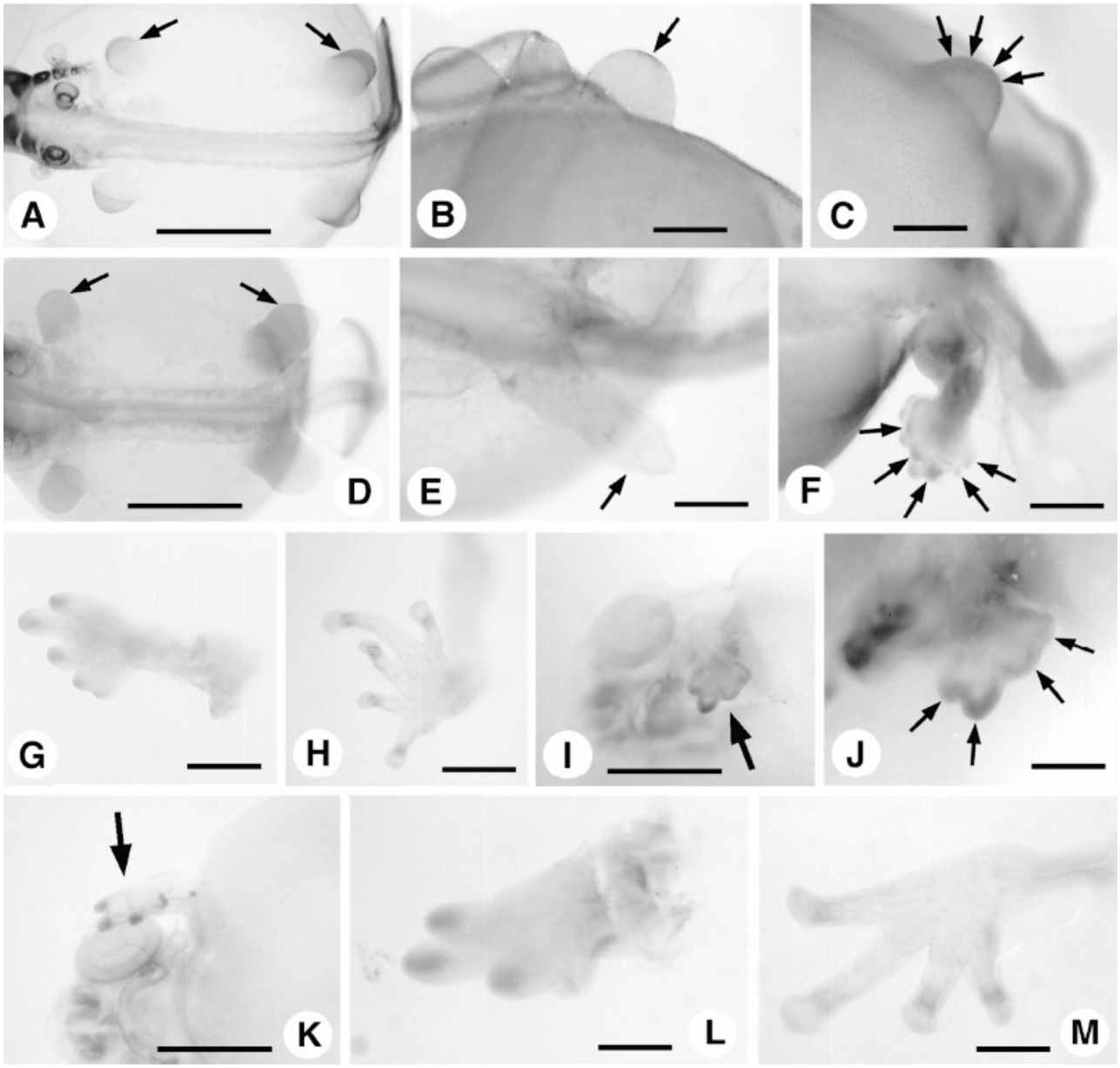

Fig. 4. Expression of *Distal-less* (Dlx) protein in embryos of *E. coqui*. **A**: Stage 5. Dorsal view; anterior is to the left. Dlx protein is expressed in fore- and hind limb buds (arrows), as well as in distal parts of the tail and in the branchial arches and cranial sensory placodes. Scale bar, 1 mm. **B**: Close-up of the hind limb buds and tail base at stage 5. Dorsal view; anterior is at the bottom. Dlx protein is expressed in the distal ectoderm (arrow). Scale bar, 0.4 mm. **C**: Stage 5. An arc of strong staining is present along the distal margin of the forelimb bud (arrows). Scale bar, 0.4 mm. **D**: Stage 6. Dorsal view; anterior is to the left. Dlx protein is expressed at low levels in each limb (arrows). Scale bar, 1 mm. **E**: Close-up of the left hind limb and tail base at stage 7. Dorsal view; anterior is to the left. Dlx protein is no longer expressed in the limbs (arrow). Faint gray shading represents nonspecific background staining. Scale bar, 0.3 mm. **F**: Close-up of the left hind limb and tail base at stage 8. Dlx protein is expressed in the digital buds (arrows). The contralateral limb is also partly visible behind the left limb. Scale bar, 0.3 mm. **G**: Dlx protein expression in the toe tips of the hind limb at stage 9. Scale bar, 0.2 mm. **H**: Hind limb, stage 12. Dlx protein is expressed in the most distal phalanx of each digit but is absent from the rudimentary toe pads. Scale bar, 0.5 mm. **I**: Ventrolateral view of the head and forelimb at stage 8; anterior is to the left. Dlx protein is expressed in the forelimb (arrow) from the earliest morphological signs of digit differentiation. Scale bar, 1 mm. **J**: Close-up of the forelimb in I. Each digital bud stains for Dlx protein (arrows), as do the interdigital areas. Scale bar, 0.3 mm. **K**: Ventrolateral view of the head and forelimb (arrow) at stage 9; anterior is to the left. All four digits express Dlx protein in the distal ectoderm and mesenchyme. Scale bar, 1 mm. **L**: Close-up of forelimb in K. Scale bar, 0.2 mm. **M**: Forelimb, stage 12. As in the hind limb, Dlx protein is present in the distal portion of each digit, but not in the adhesive toe-pads. Scale bar, 0.2 mm.

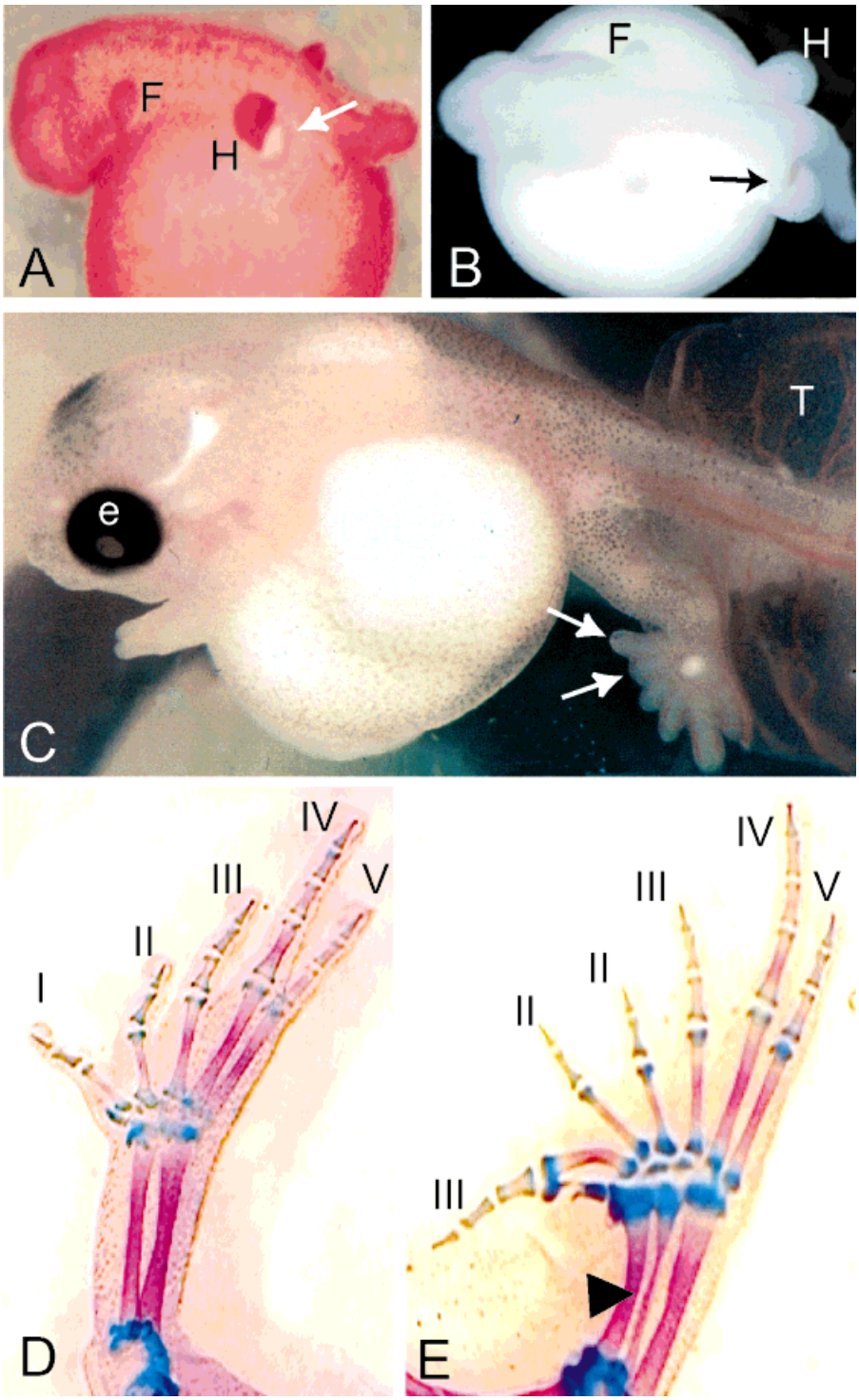

Fig. 5. ZPA transplantation. **A**: ZPA donor. Lateral view of a stage-4 embryo dyed with neutral red. The posterior portion of the left hind limb bud, which contains the presumptive zone of polarizing activity (ZPA), has been ablated (white arrow). **B**: ZPA host. The red explant from the donor embryo in A has been grafted into the hind limb bud of a second, host embryo (arrow). **C**: Lateral view of a ZPA host embryo at stage 11. Digit I is duplicated in the left hind limb (arrows), which earlier received a donor ZPA graft. **D**: Normal (control) hind limb skeleton at hatching (stage 15). **E**: Hind limb skeleton in a ZPA host at the same stage. The chimaeric host limb appears to lack digit I and instead has duplicate digits II and III. The arrow points to a supernumerary long bone in the zeugopodium. Limbs in D and E are stained with Alcian blue (cartilage) and alizarin red (bone); digits are labeled I–V.

TABLE 2. Incidence of supernumerary digits following ZPA transplantation[1]

Supernumerary digits	Stage 3 (3)	Stage 4 (7)	Stage 5 (4)
I	2	7	2
II	3	3	2
III	1	2	0

[1]In all cases, the presumptive ZPA region from the hind limb bud of a donor embryo was transplanted into the anterior portion of the corresponding limb bud of a host embryo at the same stage. Specimens were allowed to develop until stage 15, just prior to hatching, when they were preserved and stained for cartilage with Alcian blue. Values denote numbers of stage-15 host specimens that display each type of duplicated digit. Sample sizes per transplant stage are in parentheses.

Chondrogenesis in E. coqui

Overall limb chondrogenesis in *E. coqui* proceeds in a proximodistal sequence. Within the manus and pes, digits form from posterior to anterior, with digits III and IV forming first and digit I last. Both features are characteristic of limb development in metamorphosing anurans (Kemp and Hoyt, '69; Trueb and Hanken, '92) and thus appear to have been retained during the evolution of direct development in this lineage of frogs. Indeed, a posteroanterior sequence of digit formation is characteristic of all tetrapods except urodeles, in which digits form from anterior to posterior (Shubin and Alberch, '86; Stark et al., '98).

Shh *expression and the zone of polarizing activity*

Expression of the gene *Sonic hedgehog* (*Shh*) and function of the zone of polarizing activity (ZPA) are highly characteristic features of vertebrate limb development (Shubin et al., '97). *Shh* is generally expressed within the ZPA along the posterior margin of the early limb bud (Riddle et al., '93; Helms et al., '94; Niswander et al., '94; Pearse and Tabin, '98). Ablation of this region truncates limb outgrowth and differentiation (Pagan et al., '96), whereas transplantation of the ZPA to the anterior region of a host limb bud typically induces supernumerary digits (Hinchliffe et al., '81; Honig and Summerbell, '85; Summerbell, '79). Although these and other basic features of the currently accepted "model" of vertebrate limb development are derived principally from the study of amniotes (Martin, '98), metamorphosing anurans are known to share at least some of these features. For example, genes that mediate proximodistal and anteroposterior limb axis formation in amniotes, such as *Shh*, are expressed similarly in the clawed frog, *Xenopus laevis* (although genes associated with the dorsoventral axis are not, Christen and Slack, '98). Until the present study, polarizing activity of the presumptive ZPA in frogs had only been examined indirectly—in *Xenopus,* 180° rotation of distal portions of developing hind limbs induces formation of supernumerary digits (Cameron and Fallon, '77)—but this nevertheless suggested a specific region of polarizing activity.

Embryonic limb development in direct-developing *E. coqui* displays these same features in many, although not all, respects. Similarities include the expression of *Sonic hedgehog* (*EcShh*), which is localized to the ZPA region during early development (stages 5–9; Fig. 3). Moreover, ablation of this region leads to truncation of limb outgrowth and differentiation. Finally, transplantation of the presumptive ZPA to the anterior side of a host limb bud early in development (viz., stages 4–5) induces supernumerary digits.

Results in *E. coqui* differ, however, with respect to the duration of the period when the ZPA is active, or at least when the transplanted ZPA is capable of inducing supernumerary digits in a novel host environment. In the chicken embryo, a grafted ZPA can induce mirror-image duplication in a relatively late stage limb bud, i.e., after the bud shows dorsoventral flattening (Saunders and Gasseling, '68; Tickle et al., '75; Summerbell, '79). In *E. coqui*, ZPA transplants to a host limb bud after the onset of dorsoventral flattening (stage 6) do not induce mirror-image duplication, even though such duplications are readily induced in younger limbs. This apparent loss of polarizing activity in later stages may reflect the inability of the host tissue to respond to the polarizing signal, or it may reside in the transplantation process itself. Additional detailed information regarding the specific activity and function of the presumptive ZPA in metamorphosing anurans is needed to establish whether this apparent heterochronic shift (Richardson, '95) in *E. coqui* is unique to this and related direct-developing species, or instead is characteristic of anurans generally. Such information is only recently becoming available (e.g., Blanco et al., '98; Christen and Slack, '97, '98; Yokoyama et al., '98). Similarly, more data are needed from *E. coqui* to establish whether the apparently earlier cessation of ZPA activity is associated with a change in the timing of limb axis specification and commitment relative to that characteristic of other tetrapods.

Distal-less *protein expression in the distal ectoderm*

Homologs of the *Drosophila* gene *Distal-less* (*Dll*) are expressed in developing limbs and limblike structures in a wide variety of animals (Dolle et al., '92; Bulfone et al., '93; Ferrari et al., '95;; Panganiban et al., '97; Panganiban, 2000). In groups in which its activity has been well studied, such as insects, *Distal-less* mediates the initiation of limb development and further limb outgrowth (Panganiban et al., '94; Panganiban, 2000). The vertebrate *Distal-less* (*Dlx*) family is believed to consist of six to eight paralogous genes per species (Beanan and Sargent, 2000; Zerucha and Ekker, 2000); four genes have been described in *Eleutherodactylus coqui* (Fang and Elinson, '96).

In an earlier study of cranial development in *E. coqui*, Fang and Elinson noted the expression of two *Distal-less* genes, *EcDlx2* and *EcDlx4*, "at the edges of the limb buds" at embryonic stage 6 ('96: 166). We extend their report by showing that *Distal-less* protein is present beginning much earlier, as soon as the limb buds become morphologically distinct. Moreover, and as suggested by Fang and Elinson, the region of expression corresponds precisely to the location of the apical ectodermal ridge (AER) of most other limbed vertebrates, even though a morphologically distinct AER is absent in *E. coqui* (Richardson et al., '98). By stage 7, Dlx staining is lost in the developing limb buds, but it reappears during the onset of digit differentiation and is retained until stage 13. During this later period of embryonic expression, Dlx is expressed within mesenchyme that is differentiating to form the cartilaginous limb skeleton. This expression profile suggests two possible roles for *Dlx* during limb development in *E. coqui*: generation of the initial proximodistal axis, and regulation of chondrogenic differentiation. Both roles have been proposed for *Dlx* during limb development in amniotes (Ferrari et al., '95).

Absence of an AER in *E. coqui* is unique among anurans (or at least the few species for which data are available), although it is a characteristic feature of urodeles (Hanken, '86; Richardson et al., '98). Moreover, absence of an AER is, in each case, correlated with functional differences. Removal of the AER causes a truncation of limb development in amniotes (Summerbell, '74; Niswander et al., '93; Vogel and Tickle, '93) and in *Xenopus* (Tschumi, '57), whereas removal of the distal ectoderm does not truncate limb development in either *E. coqui* or urodeles (Lauthier, '85; Richardson et al., '98). The distal ectoderm in *E. coqui* does express Dlx, which is characteristically found within the AER of amniotes and other vertebrates. Thus, whereas the distal limb bud ectoderm is similar in both *E. coqui* and amniotes in at least one key molecular feature, Dlx expression, this important area of limb outgrowth and differentiation otherwise differs both morphologically and functionally between the two groups. Presence of an AER is not an essential requirement for limb development in all tetrapods (Richardson, '99).

Evolution of limb development in direct-developing Eleutherodactylus

Limbs offer perhaps the most obvious example of the dramatic changes to ontogeny that may accompany the evolution of direct development in frogs. A largely postembryonic event in metamorphosing species, limb development is completed before hatching in direct developers. When examined in more detail, however, limb development in the direct-developing frog *Eleutherodactylus coqui* presents a mosaic of both conserved and novel features. The former include the basic sequence and pattern of limb chondrogenesis, which are typical of anurans generally and appear largely unaffected by the gross shift in developmental timing; expression of *Distal-less* protein (Dlx) in the distal ectoderm; expression of the gene *Sonic hedgehog* (*Shh*) in the zone of polarizing activity (ZPA); and the ability of the ZPA to induce supernumerary digits when transplanted to the anterior region of an early host limb bud. Novel features include the absence of a morphologically distinct AER, the ability of the limb to continue distal outgrowth and differentiation following removal of the distal ectoderm, and earlier cessation of the inductive ability of the ZPA. The first two of these can, at this time, be viewed as coincident with the evolution of direct development, although only in frogs. Not enough is known about the ZPA in metamorphosing anurans to suggest whether the early cessation of inductive ability seen in *E. coqui* represents a third derived feature associated with direct development, or instead a retained ancestral condition.

Tetrapod limb development as module

Mechanisms that mediate limb development generally are regarded as highly conserved among tetrapod vertebrates (Raff, '96; Shubin et al., '97). Because of this fundamental conservation of underlying processes, combined with the discrete localization of the limb bud and the semi-autonomous

nature of its development, the tetrapod limb is frequently cited as an example of a developmental module (Bolker, 2000; Raff, '96; von Dassow and Munro, '99). Nevertheless, and as discussed previously, many species display considerable variation in important parameters of limb development (Richardson, '99). Specifically, the model of limb development derived principally from the study of amniotes (Tickle, '95, '96; Niswander, '96; Johnson and Tabin, '97; Martin, '98) does not apply in every important respect to all vertebrates, or even to all tetrapods.

Does this variation deny the validity of tetrapod limb development as module? We think not. It does reveal, however, that modules in living organisms are not static; they can and do evolve. Moreover, variation in the mechanisms that underlie limb development in tetrapods is structured. In some instances, the variation displays a phylogenetic component; that is, one or more variant features are shared by all members of a given lineage as a result of their common ancestry. In others, variation can be associated with an adaptive change in life history or reproductive mode. A major challenge remains to define the extent to which these differences in developmental mechanism facilitate or constrain the diversification of limb morphology in individual clades.

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LITERATURE CITED

Akimenko MA, Ekker M. Anterior duplication of the Sonic hedgehog expression pattern in the pectoral fin buds of zebrafish treated with retinoic acid. Dev Bio 170:243–247.

Ancel LW, Fontana W. 2000. Plasticity, evolvability, and modularity in RNA. J Exp Zool (Mol Dev Evol) 288:242–283.

Beanan MJ, Sargent TD. 2000. Regulation and function of *Dlx3* in vertebrate development. Dev Dynam 218:545–553.

Blanco MJ, Misof BY, Wagner GP. 1998. Heterochronic differences of *Hoxa-11* expression in *Xenopus* fore- and hind limb development: evidence for lower limb identity of the anuran ankle bones. Dev Genes Evol 208:175–187.

Bolker JA. 2000. Modularity in development and why it matters to evo-devo. Am Zool 40:770–776.

Bulfone A, Kim HJ, Puelles L, Porteus MH, Grippo JF, Rubenstein JLR. 1993. The mouse *Dlx-2 (Test-1)* gene is expressed in spatially restricted domains of the forebrain, face and limbs in midgestation mouse embryos. Mech Dev 40:129–140.

Burian RM. 2000. General introduction to the symposium on evolutionary developmental biology: paradigms, problems, and prospects. Am Zool 40:711–717.

Callery EM, Elinson RP. 2000. Opercular development and ontogenetic re-organization in a direct-developing frog. Dev Genes Evol 210:377–381.

Callery EM, Fang H, Elinson RP. 2001. Frogs without polliwogs: Evolution of anuran direct development. BioEssays 23:233–241.

Cameron J, Fallon JF. 1977. Evidence for polarizing zone in the limb buds of *Xenopus laevis*. Dev Biol 55:320–330.

Carl TF, Klymkowsky MW. 1999. Visualizing endogenous and exogenous proteins in *Xenopus* (and other organisms). In: Richter J, editor. A comparative methods approach to the study of oocytes and embryos. New York: Oxford Press, p 291–315.

Carroll SB. 2001. Chance and necessity: the evolution of morphological complexity and diversity. Nature 409:1102–1109.

Christen B, Slack JM. 1997. FGF-8 is associated with anteroposterior patterning and limb regeneration in *Xenopus*. Dev Biol 192:455–466.

Christen B, Slack JM. 1998. All limbs are not the same. Nature 395:230–231.

Dent JA, Polson AG, Klymkowsky MW. 1989. A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in *Xenopus*. Development 105:61–74.

Dolle P, Price M, Duboule D. 1992. Expression of the murine Dlx-1 homeobox gene during facial, ocular and limb development. Differentiation 49:93–99.

Elinson RP. 1987. Changes in developmental patterns: embryos of amphibians with large eggs. In: Raff RA, Raff EC, editors. Development as an evolutionary process. New York: Alan R. Liss, Inc., p 1–21.

Elinson RP. 1990. Direct development in frogs: wiping the recapitulationist slate clean. Sem Dev Biol 1:263–270.

Elinson RP. 1994. Leg development in a frog without a tadpole (*Eleutherodactylus coqui*). J Exp Zool 270:202–210.

Elinson RP. 2001. Direct development: An alternative way to make a frog. Genesis: J Gen Dev 29:91–95.

Elinson RP, del Pino EM, Townsend DS, Cuesta FC, Eichhorn P. 1990. A practical guide to the developmental biology of terrestrial-breeding frogs. Biol Bull 179:163–177.

Fang H, Elinson R. 1996. Patterns of Distal-less gene expression and inductive interactions in the head of the direct developing frog *Eleutherodactylus coqui*. Dev Biol 179: 160–172.

Ferrari D, Sumoy L, Gannon J, Sun H, Brown AM, Upholt WB, Kosher RA. 1995. The expression pattern of the *Distal-less* homeobox-containing gene *Dlx-5* in the developing chick limb bud suggests its involvement in apical ectodermal ridge activity, pattern formation, and cartilage differentiation. Mech Dev 52:257–264.

Gardiner DM, Blumberg B, Komine Y, Bryant SV. 1995. Regulation of *HoxA* expression in developing and regenerating axolotl limbs. Development 121:1731–1741.

Gerhart J, Kirschner M. 1997. Cells, embryos and evolution. Oxford: Blackwell Scientific.

Hamburger V. 1960. A manual of experimental embryology. Revised edition. University of Chicago Press, Chicago.
Hall BK. 2000. Balfour, Garstang de Beer: the first century of evolutionary embryology. Am Zool 40:718–728.
Hanken J. 1986. Developmental evidence for amphibian origins. In: Hecht MK, Wallace B, Prance GT, editors. Evolutionary biology, vol. 20. New York: Plenum Press, p 389–417.
Hanken J. 1992. Life history and morphological evolution. J Evol Biol 5:549–557.
Hanken J. 1999. Larvae in amphibian development and evolution. In: Hall BK, Wake MH, editors. The origin and evolution of larval forms. San Diego: Academic Press, p 61–108.
Hanken J, Klymkowsky MW, Summers CH, Seufert DW, Ingebrigtsen N. 1992. Cranial ontogeny in the direct-developing frog, *Eleutherodactylus coqui* (Anura: Leptodactylidae), analyzed using whole-mount immunohistochemistry. J Morphol 211:95–118.
Harland RM. 1991. In situ hybridization: an improved whole-mount method for *Xenopus* embryos. In: Kay BK, Peng HB, editors. *Xenopus laevis*: practical uses in cell and molecular biology. Methods in cell biology, vol. 36. Academic Press, San Diego, p 685–695.
Hartwell LH, Hopfield JJ, Liebler S, Murray AW. 1999. From molecular to modular cell biology. Nature 402:C47–C52.
Helms J, Thaller C, Eichele G. 1994. Relationship between retinoic acid and Sonic hedgehog, two polarizing signals in the chick wing bud. Development 120:3267–3274.
Hemmati-Brivanlou A, Frank D, Bolce ME, Brown BD, Sive HL, Harland RM. 1990. Localization of specific mRNAs in *Xenopus* embryos by whole-mount in situ hybridization. Development 110:325–330.
Hinchliffe JR, Garcia-Porrero JA, Gumpel-Pinot M. 1981. The role of the zone of polarizing activity in controlling the differentiation of the apical mesenchyme of the chick wing-bud: histochemical techniques in the analysis of a developmental problem. Histochem J 13:643–658.
Hinchliffe JR, Johnson DR. 1980. The development of the vertebrate limb. Oxford: Clarendon Press.
Honig LS, Summerbell D. 1985. Maps of strength of positional signalling activity in the developing chick wing bud. J Embryol Exp Morphol 87:163–174.
Johnson RL, Tabin CJ. 1997. Molecular models for vertebrate limb development. Cell 90:979–990.
Kemp NE, Hoyt JA. 1969. Sequence of ossification in the skeleton of growing and metamorphosing tadpoles of *Rana pipiens*. J Morphol 129:415–443.
Kirschner M, Gerhart J. 1998. Evolvability. Proc Natl Acad Sci USA 95:8420–8427.
Klingenberg CP, Zaklan SD. 2000. Morphological integration between developmental compartments in the *Drosophila* wing. Evolution 54:1273–1285.
Klymkowsky MW, Hanken J. 1991. Whole-mount staining of Xenopus and other vertebrates. Meth Cell Biol 36:419–441.
Laforest L, Brown CW, Poleo G, Geraudie J, Tada M, Ekker M, Akimenko MA. 1998. Involvement of the *Sonic hedgehog, patched 1* and *bmp2* genes in patterning of the zebrafish dermal fin rays. Development 125:4175–4184.
Lauthier M. 1985. Morphogenic role of epidermal and mesodermal components of the fore- and hindlimb buds of the newt *Pleurodeles waltlii* Michah. (Urodela, Amphibia). Arch Biol (Bruxelles) 96:23–43.
Mahmood R, Bresnick J, Hornbruch A, Mahony C, Morton N, Colquhoun K, Martin P, Lumsden A, Dickson C, Mason I. 1995. A role for *Fgf-8* in the initiation and maintenance of vertebrate limb bud outgrowth. Curr Biol 5:797–806.
Martin GR. 1998. The role of FGFs in the early development of vertebrate limbs. Genes Dev 12:1571–1586.
Niswander L. 1996. Growth factor interactions in limb development. Ann New York Acad Sci 785:23–26.
Niswander L, Jeffrey S, Martin GR, Tickle C. 1994. A positive feedback loop coordinates growth and patterning in the vertebrate limb. Nature 371:609–612.
Niswander L, Tickle C, Vogel A, Booth I, Martin GR. 1993. FGF-4 replaces the apical ectoderm ridge and directs outgrowth and patterning of the limb. Cell 75:579–587.
Olsson L, Hanken J. 1996. Cranial neural crest migration and chondrogenic fate in the Oriental fire-bellied toad, *Bombina orientalis*: defining the ancestral pattern of head development in anuran amphibians. J Morphol 229:1–16.
Pagan SM, Ros MA, Tabin C, Fallon JF. 1996. Surgical removal of limb bud Sonic hedgehog results in posterior skeletal defects. Dev Biol 180:35–40.
Panganiban G. 2000. *Distal-less* function during *Drosophila* appendage and sense organ development. Dev Dynam 218:554–562.
Panganiban G, Irvine SM, Lowe C, Roehl H, Corley LS, Sherbon B, Grenier JK, Fallon JF, Kimble J, Walker M, Wray GA, Swalla BJ, Martindale MQ, Carroll SB. 1997. The origin and evolution of animal appendages. Proc Natl Acad Sci USA 94:5162–5166.
Panganiban G, Nagy L, Carroll SB. 1994. The role of the *Distal-less* gene in the development and evolution of insect limbs. Curr Biol 4:671–675.
Pearse RV, Tabin CJ 1998. The molecular ZPA. J Exp Zool 282:677-690.
Presnell JK, Schreibman MP. 1997. Humason's animal tissue techniques, 5th ed. Baltimore: The Johns Hopkins University Press.
Raff RA. 1996. The shape of life: genes, development, and the evolution of animal form. Chicago: The University of Chicago Press.
Raff RA, Wray GA. 1989. Heterochrony: developmental mechanisms and evolutionary results. J Evol Biol 2:409–434.
Richardson MK. 1995. Heterochrony and the phylotypic period. Dev Biol 172:412–421.
Richardson MK. 1999. Vertebrate evolution: the developmental origins of adult variation. BioEssays 21:604–613.
Richardson MK, Carl TF, Hanken J, Elinson RP, Cope C, Bagley P. 1998. Limb development and evolution: a frog embryo with no apical ectodermal ridge (AER). J Anat 192:379–390.
Riddle RD, Johnson RL, Laufer E, Tabin C. 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75: 1401–1416.
Saunders JW Jr. 1948. The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. J Exp Zool 108:363–404.
Saunders JW Jr., Gasseling MT. 1968. Ectodermal-mesodermal interactions in the origin of limb symmetry. In: Fleischmajer R, Billingham RE, editors. Epithelial mesenchymal interactions. Baltimore: Williams and Wilkins, p 78–97.
Schauerte HE, van Eeden FJ, Fricke C, Odenthal J, Strahle U, Haffter P. 1998. Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish. Development 125:2983–2993.
Schlosser G. 2001. Using heterochrony plots to detect the dissociated coevolution of characters. J Exp Zool (Mol Dev Evol) 291:282–304.
Schlosser G, Roth G. 1997. Evolution of nerve development

in frogs: II. Modified development of the peripheral nervous system in the direct-developing frog *Eleutherodactylus coqui* (Leptodactylidae). Brain Behav Evol 50:94–128.

Schlosser G, Thieffry D. 2000. Modularity in development and evolution. BioEssays 22:1043–1045.

Shubin N, Tabin C, Carroll S. 1997. Fossils, genes and the evolution of animal limbs. Nature 388:639–648.

Shubin NH, Alberch P. 1986. A morphogenetic approach to the origin and basic organization of the tetrapod limb. In: Hecht MK, Wallace B, Prance G, editors. Evolutionary biology, vol. 20. New York: Plenum Press, p 319–388.

Stark DR, Gates PB, Brockes JP, Ferretti P. 1998. Hedgehog family member is expressed throughout regenerating and developing limbs. Dev Dynam 212:352–363.

Summerbell D. 1974. A quantitative analysis of the effect of excision of the AER from the chick limb-bud. J Embryol Exp Morphol 32:651–660.

Summerbell D. 1979. The zone of polarising activity: evidence for a role in normal chick limb morphogenesis. J Embryol Exp Morphol 50:217–233.

Tarin D, Sturdee AP. 1971. Early limb development of *Xenopus laevis*. J Embryol Exp Morphol 26:169–179.

Tickle C. 1995. Vertebrate limb development. Curr Opin Gen Dev 5:478–484.

Tickle C. 1996. Genetics and limb development. Dev Gen 19:1–8.

Tickle C, Summerbell D, Wolpert L. 1975. Positional signaling and specification of digits in chick limb morphogenesis. Nature 254:199–202.

Torok MA, Gardiner DM, Shubin NH, Bryant SV. 1998. Expression of HoxD genes in developing and regenerating axolotl limbs. Dev Biol 200:225–233.

Townsend DS, Stewart MM. 1985. Direct development in *Eleutherodactylus coqui* (Anura: Leptodactylidae): a staging table. Copeia 1985:423–436.

Trueb L, Hanken J. 1992. Skeletal development in *Xenopus laevis* (Anura: Pipidae). J Morphol 214:1–41.

Tschumi PA. 1957. The growth of the hindlimb bud of *Xenopus laevis* and its dependence upon the epidermis. J Anat 91:149–172.

Vogel A, Tickle C. 1993. *Fgf-4* maintains polarizing activity of posterior limb bud cells in vivo and in vitro. Development 119:199–206.

von Dassow G, Munro E. 1999. Modularity in animal development and evolution: elements of a conceptual framework for evodevo. J Exp Zool (Mol Dev Evol) 285:307–325.

Wagner GP, Altenberg L. 1996. Complex adaptations and the evolution of evolvability. Evolution 50:967–976.

Yokoyama H, Endo T, Tamura K, Yajima H, Ide H. 1998. Multiple digit formation in *Xenopus* limb bud recombinants. Dev Biol 196:1–10.

Zerucha T, Ekker M. 2000. *Distal-less*-related homeobox genes of vertebrates: evolution, function, and regulation. Biochem. Cell Biol 78:593–601.